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Isolation, Characterization and Antimicrobial Activity of 3β, 22E-Stigmasta-5, 22-dien-3-ol from the Aerial Part of *Aeschynomene uniflora* E. Mey

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Authors' contributions

This work was carried out in collaboration between all authors. All authors read and approved the final manuscript.

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Original Research Article

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ABSTRACT

Objectives: To isolate, characterize and determine the antimicrobial activity of bioactive compounds present in the petroleum ether fraction of the aerial parts of *Aeschynomene uniflora* E. Mey.
Methods: *Aeschynomene uniflora* was collected, identified, dried and pulverized. The pulverized plant material was subjected to Soxhlet extraction using petroleum ether. The petroleum ether extract was subjected to chromatographic techniques which yielded J1. J1 was then subjected to antimicrobial screening using some selected microorganisms. J1 was characterized using IR and NMR spectroscopic techniques.
Results: The structure of the isolated compound was established to be 3β, 22E-Stigmasta-5, 22-dien-3-ol (stigmasterol) using spectroscopic analysis (IR, 1D-NMR). The isolated compound showed significant antimicrobial activity on some selected microorganisms.
Conclusion: The results could justify the use of the aerial parts of *Aeschynomene uniflora* in traditional herbal medicine for the treatment of various microbial diseases.

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Keywords: Aeschynomene uniflora; aerial part; stigmasterol and antimicrobial activity.

1. INTRODUCTION

Extraction, identification and isolation of chemical substances have now formed the major field of study in chemical and pharmaceutical sciences. This may be due to the wide spread of drug resistant microorganisms [1]. These investigations have been triggered by the emergence and spread of antibiotic resistant microorganism causing the effective life-span of existing antibiotics to be limited. Hence the plant kingdom is being screened for newer and effective chemotherapeutic agents. Higher plants can serve both as potential antimicrobial crude drugs as well as source of new antiinfective agents [2]. *Aeschynomene uniflora* belongs to the family Fabaceae. It is an erect or ascending, rarely almost prostate, annual or short-lived plant found in several places in Africa, especially in fresh water swamp and aquatic vegetation [3]. The plant is used in traditional medicine for treatment of psychotic disorder, tuberculosis, skin infection, antidote to snake venom, menstrual disorder and small pox. The aqueous extract of the whole plant is administered topically over the whole body to cure small pox in northern Nigeria. The plant is eaten as vegetable to cure fever symptoms and cough in Benue state Nigeria. This study was aimed at isolation and characterization of bioactive principles from the aerial parts of *Aeschynomene uniflora* Mey.

2. EXPERIMENTAL SECTION

2.1 Collection, Identification and Preparation of Plant Materials

The aerial parts of *Aeschynomene uniflora* (Fabaceae) was collected from Benue State, Nigeria, in the month of July, 2012. It was authenticated by Mr. U.S Galla of Herbarium unit, Department of Biological Science, Ahmadu Bello University, Zaria, Nigeria and a voucher specimen, number 2408, was deposited for future reference. The plant material was air-dried, pulverized and store in paper bags prior to extraction.

2.2 Extraction and Isolation

The pulverized plant material (200 g) extracted using petroleum ether (60-80℃). The crude extract was respectively concentrated in vacuo at 40℃ using a rotary evaporator [4]. The recovery yeild petroleum ether extract was 7 g.

2.3 Chromatographic Separation

The petroleum ether extract of *A. uniflora* was subjected to TLC (Thin Layer Chromatography) using silica gel as stationary phase and petroleum ether : ethyl acetate (9:1) as well as petroleum ether : chloroform, 7:3 as mobile phase. When the chromatograms where sprayed with 10% H_2SO_4 and heated slightly, they yielded five spots. A column of length 40 cm in length and 3.5 cm in diameter was used for the column chromatography of the petroleum ether extract (7 g). 40 g of Silica gel (60 - 120 mesh) was used as stationary phase. Petroleum ether : ethyl acetate, 97.5 : 2.5, 95 : 5 and 92.5 : 7.5 was used as mobile phase by gradient elution technique. A total of 60 eluates of 100 mL each were collected and combined together base on their TLC profile. 7 fractions were obtained and labeled as F1, F2, F3, F4, F5, F6 and F7. Elutes 28 – 34 (F5), which were eluted using a mobile phase of petroleum ether : ethyl acetate, 95 : 5, were further purified using preparative TLC (and a solvent system of petroleum ether : ethyl acetate, 97.5 : 2.5) which gave rise to a white powdery substance (J1). The J1 gave a single spot when subjected to TLC using solvent systems including petroleum ether : ethyl acetate (90:10) and petroleum ether : chloroform : methanol (60:30:10). J1 was a white crystalline powder (9 mg) with a melting point 168 – 169℃. J1 was further subjected to spectroscopic analysis such as infrared spectroscopy (IR), nuclear magnetic resonance spectroscopy (^{1}H NMR and ^{13}C NMR) to ascertain the chemical structure of the compound.

2.4 The Antimicrobial Screening

The antimicrobial activity of the compound was determined using agar well diffusion method. The microorganisms were obtained from the Department of Medical Microbiology ABU Teaching Hospital Zaria. Ciprofloxacin and fluconazole (60 µg/mL) were used as positive standards control drugs. 600 µg of the isolated compound was weighed and dissolved in 10 mL DMSO to obtain a concentration of 60 µg/mL of the compound; this was the initial concentration of the compound used to determine the antimicrobial activity of the compound. Mueller Hinton agar was prepared according to the manufacturer's instruction, sterilized at 121ºC for 15 minutes; the sterilized medium was then poured into sterile petri dishes. The plates were

allowed to cool and solidify. The sterilized medium was seeded with 0.1 mL of the standard inoculum of the test microorganism; the inoculum was spread evenly over the surface of the medium with a sterile swab. Wells were bored into the solidified inoculated using a standard broth borer of 6 mm in diameter. 0.1 mL of the solution of the compound of concentration 60 µg/mL was then introduced into each well on the medium. The inoculated medium was then incubated at 37ºC for 24 h after which each plate was observed for the zone of inhibition of growth which was measured with a transparent ruler and the result recorded in millimeters.

2.5 Minimum Inhibitory Concentration Assay

The minimum inhibition concentration (MIC) assay of the compound was carried out using broth dilution method. Mueller Hinton broth was prepared, of which 10 mL was dispensed into test tubes and sterilized at 121ºC for 15 minutes and the broth was allowed to cool. Mc-Farland turbidity standard scale number 0.5 was prepared to give turbid solution. Normal saline was prepared, 10 mL was dispensed into sterile test tubes and the test microbes were inoculated, incubated at 37ºC for 6 hours. Dilution of the test microbes was done in the normal saline until the turbidity march that of the Mc- Farland's scale by visual comparison, at this point the test microbes has a concentration of about 1.5 X 10^8 *cfu*/mL. Two-fold serial dilution of the compound in the sterile broth was made to obtain the concentration of 60 µg/mL, 30 µg/mL, 15 µg/mL L, 7.5 µg/mL and 3.25 µg/mL. 0.1 ml of the standard inoculums of the test microbes microorganism in the normal saline was then inoculated in to the different concentrations, incubations was made at 37ºC for 24 h, after which each test tube of the broth was observed for turbidity (growth). The lowest concentration of the compound in the broth which shows no turbidity was recorded as the minimum inhibition concentration [5,6].

2.6 Minimum Bactericidal Concentration/ Minimum Fungicidal Concentration Assays

The minimum bactericidal concentration / minimum fungicidal concentration (MBC/MFC) was carried out to determine if the test microbes were killed or only their growth was inhibited. Mueller-Hinton agar was prepared and sterilized at 121ºC for 15 minutes, poured into petri dishes and were allowed to cool and solidify. The content of the MIC in the serial dilution was sub cultured onto the prepared medium and incubation was done at 37ºC for 24 h. Thereafter, each plate of the medium was observed for colony growth. The value obtained in the plate with lowest concentration of the compound without colony growth was recorded as the MBC/MFC [5,6].

2.7 Equipment

(I). The melting point of the compound was determined using Ernst Leitz Wetzlar melting point machine.

(II). FTIR Spectroscopy (Shimadzu FTIR8 400s Fourier Transform Infra-Red Spectroscopy).

(III). NMR spectra were obtained on a Bruker AVANCE spectrometer (600 MHz), using the TMS peaks as standard.

3. RESULTS AND DISCUSSION

The IR spectrum of J1, showed a very intensely broad band absorption peak at 3416 cm^{-1} for the O-H bond vibrations of hydroxyl group. A moderate intense band at 2933 cm^{-1} is assigned to aliphatic C-H stretch and at 2359.39 cm^{-1} a weak C-O stretch was observed. The C = C vibration was shown around 1732.13 cm^{-1} as weakly intense band and a corresponding out of plane C-H vibrations of the unsaturated part was observed at 1062.81 cm^{-1}. These assignments were in good agreement with reported values [7,8]. H3 proton appeared as a triplet of a double doublet (tdd) at 3.51 ppm (tdd, 1H, *J* = 4.5, 4.2, 3.8 Hz) and H6 proton signal appeared at δ 5.36 ppm (*J* 5.2 Hz, H6). These two proton typical of a steroidal nucleus. Two olefinic protons appeared as characteristic downfield signals at δ 5.16 (1H, dd, *J* = 15.0, 6.5 Hz) and 5.03 (1H, dd, *J* = 15.0, 9.0 Hz) in the ^{1}H NMR spectrum which were identical with the chemical shift of H22 and H23 respectively of stigmasterol The spectrum also displayed two proton singlets at δ 1.00 and δ 0.67 assignable for H19 and H18 respectively. In addition, two doublets at δ 0.82 (3H, d, 7.2 Hz) and 0.80 (3H, d, 7.2 Hz) could be ascribed to the two methyl groups at H26 and H27 and another three-proton doublet at δ 0.91 (3H, d, 6.8 Hz) for H21. On the other hand, one three-proton triplet at δ0.85 (3H, t, 7.2 Hz) could be assigned to the primary methyl group attached H29 [9,10]. Six methyl protons also appeared at 1.21, 1.17, 1.03, 0.99, 0.97 and 0.90 ppm. These assignments were in good agreement with reported values

[10,11]. The ^{13}C NMR has shown recognizable signals at 140.8 and 121.7 ppm, which are assigned C5 and C6 double bonds respectively. The chemical shift value at 71.0 ppm is due to C3 hydroxy group [12]. The signals at 19.4 and 11.9 ppm correspond to carbon atom (C19 and C18 respectively). The value for C18 was lower due to -gauche interaction that increases the screening of the C18 hence lower chemical shift. [13]. DEPT experiment revealed the presence of six methyl carbons at C18, C19, C21, C26, C27, and C29; nine methylene carbons at C1, C2, C4, C7, C11, C12, C15, C16 and C28; eleven methine carbons at C3, C6, C8, C9, C14, C17, C20, C22, C23, C24 and C25 and three quaternary carbons at C5, C10 and C13. The de-shielded signal at 71.8 was due to C3 with a hydroxyl group attached to it. Based on the evidence of IR, ^{1}H NMR, ^{13}C NMR and a comparison of the ^{13}CNMR signal with those described in the literatures [14-19], the isolated compound was identified as stigmasterol. The chemical structure of stigmasterol is shown in Fig. 1.

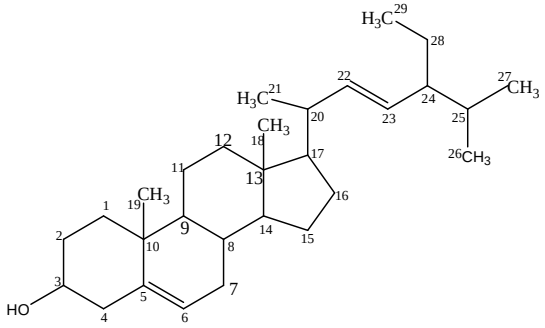


Fig. 1. J1: 3β, 22E-Sigmasta-5, 22-dien-3-ol ($C_{29}H_{48}O$, 412.7 g/mol)

3.1 Antimicrobial Activity of J1

The antimicrobial activities of the isolated compound using agar diffusion method and ten microbial species was carried out: Methicillin Resistant *Staphylococcus Aureus, Candida albicans, Candida stellatoidea, Candida krusei, Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Proteus mirabilis, Staphloccoccus aureus,* and *Streptococcus pyogenes.* The activities of the compound were expressed in terms of growth inhibition zones (given in mm). The isolated compound J1 was found to be effective against: *C. stellatoidea, Escherichia coli, Candida albicans,* Methicillin Resistant *Staphylococcus Aureus*, *Staphloccoccus aureus, Proteus mirabilis and Klebsiella pneumoniae* with a zone of inhibition of 25, 25, 27, 28, 30, 31 and 34 mm respectively. The demonstration of broad spectrum activity of stigmasterol against the test microorganism provides scientific basis for the local application of this plant in the treatment of various ailments. This observation is very significant because of the possibility of developing therapeutic substances that will be active against multidrug resistant organisms. Plant based product have been effectively proven for their utilization as source for antimicrobial compound [20]. The minimum inhibitory concentration (MIC) showed that J1 inhibited the growth of MRSA, *S. aureus, P. mirabilis and K. pneumoniae* at a concentration of 3.2 µg/mL. At a concentration of 7.5 µg/mL, the growth of *C. stellatoidea, E. coli,* and *C.albicans* were inhibited. The minimum bactericidal/fungicidal concentrations (MBC/MFC) was found to be 7.5 µg/mL against *S. aureus, P. mirabilis* and *K. pneumoniae,* while the other microbes were inhibited at the values of 15 µg/mL. The significant activity shown by the isolated compound is beneficial as it indicates probably the emergence of a new antibiotic with such a wide spectrum of activity. The fact that the treatment of infection caused by the test organisms is becoming difficult further strengthens the importance of these findings and the need for a continuous search for chemotherapeutic agents.

Table 1. Infra-red table

Serial no.	Frequency, cm^{-1}	Bond	Functional group
1	1062.81	C-H	Alkenes
2	1381.08	C-Os	Alcohols
3	1464.02	C–H bend	Alkanes
4	1732.13	C=C	Alkenes
5	2359.02	C-O sharp stretch	Alcohols
6	2864.39	C-H sharp stretch	Alkanes
7	2933.83	C–H stretch	Alkanes
8	3416.05	O–H stretch, H–bonded	Alcohols

Table 2. Comparing the ^{13}CNMR and ^{1}HNMR spectral data of "J1" with that obtained from the literature

S/No	Position	CHn	^{13}C (ppm)	^{13}C [21]	δ_H (JI)	δ_H
1	C1	CH_2	37.4	37.3	1.90	1.90
2	C2	CH_2	31.7	31.6	1.56	1.53
3	C3	CH	71.8	71.8	3.53	3.54
4	C4	CH_2	42.4	42.3	2.30	2.30
5	C5	C	140.8	140.8		
6	C6	CH	121.8	121.7	5.40	5.39
7	C7	CH_2	31.7	31.9	1.50	1.51
8	C8	CH	31.7	31.9	2.30	1.98
9	C9	CH	51.1	51.2	0.98	0.98
10	C10	C	36.6	36.5		
11	C11	CH_2	21.1	21.1	1.50	1.28
12	C12	CH_2	39.6	39.7	2.06	2.06
13	C13	C	42.3	42.3		
14	C14	CH	56.9	56.9	1.04	1.04
15	C15	CH_2	24.3	24.4	1.11	1.28
16	C16	CH_2	28.4	28.4	1.30	1.30
17	C17	CH	56.0	56.1	1.16	1.05
18	C18	CH_3	11.0	11.0	1.00	1.00
19	C19	CH_3	21.1	21.2	0.67	0.77
20	C20	CH	40.5	40.5	1.40	2.00
21	C21	CH_3	21.2	21.2	0.91	0.93
22	C22	CH	138.3	138.3	5.16	5.16
23	C23	CH	129.3	129.3	5.05	5.03
24	C24	CH	51.2	51.2	0.97	0.97
25	C25	CH	31.9	31.9	1.71	1.74
26	C26	CH_3	21.2	21.2	0.82	0.81
27	C27	CH_3	19.1	19.0	0.80	0.80
28	C28	CH_2	25.4	25.4	1.31	1.31
29	C29	CH_3	12.1	12.1	0.89	0.89

In this study, the antimicrobial activities were compared with standard antimicrobial agents, ciprofloxacin and fluconazole, which were used as positive controls. The diameters of zone of inhibition of J1 ranged from 25 to 34 mm and were lower than those of the standard antibiotics, ranging from 30 to 40 mm. J1 was less potent than the standard antibiotics. The result obtained in the present study could justify for the first time the use of the aerial parts of *A. uniflora* in the traditional medicine for the treatment of microbial diseases especially those caused by MRSA, *S. aureus, P. mirabilis, and K. pneumoniae.*

Table 3. Zones of inhibition of J1 against the test microorganism

Test organism	Zone of inhibition (mm)		
	J1	Ciprofloxacin	Fluconazole
MRSA	28	0	0
C. albicans	27	0	35
C. stellatoidea	25	0	32
C. krusei	0	0	37
E. coli	25	35	0
K. pneumonia	34	40	0
P. aeruginosa	0	32	0
P. mirabilis	31	30	0
S. aureus	25	35	0
S. pyogenes	0	37	0

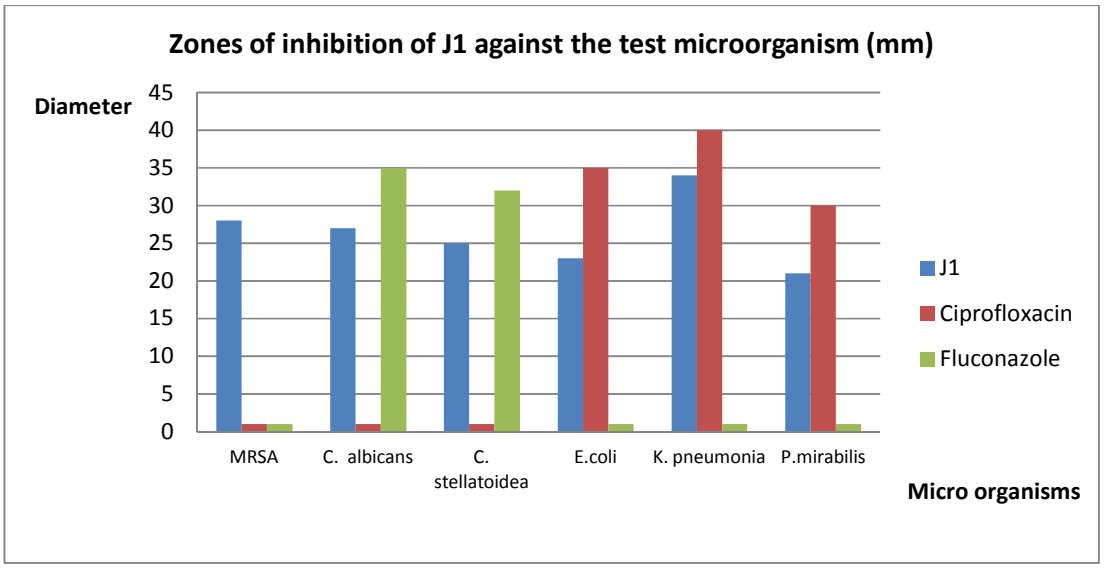
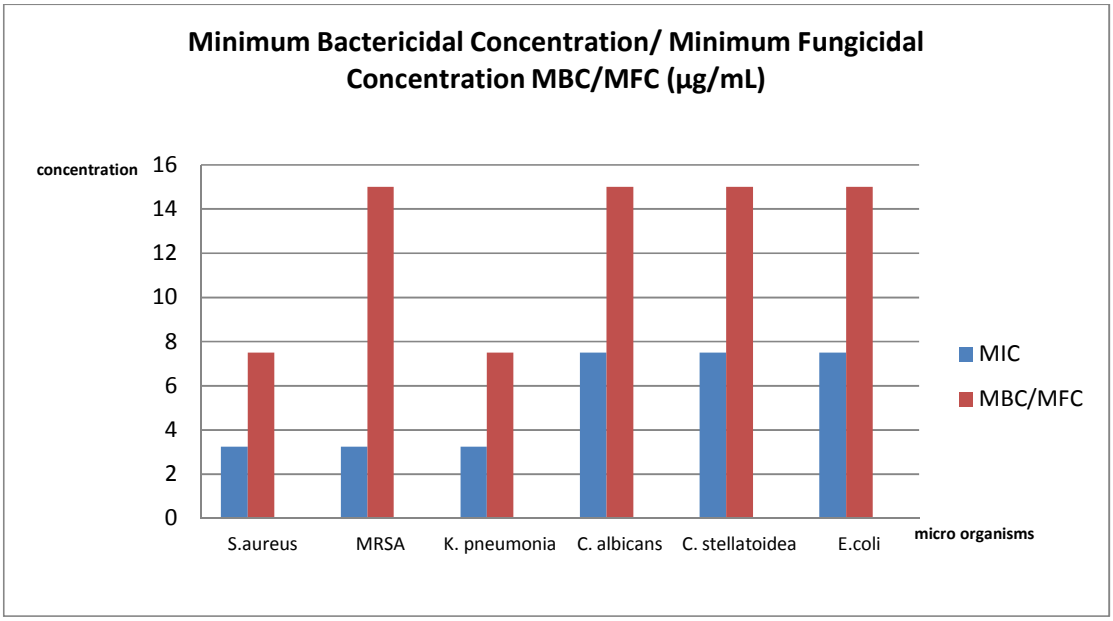


Table 4. Minimum Bactericidal Concentration/ Minimum Fungicidal Concentration MBC/MFC (µg/mL)

Test organism	MIC	MBC/MFC
	J1	J1
S. aureus	3.25	7.5
MRSA	3.25	15
K. pneumonia	3.25	7.5
C. albicans	7.5	15
C. stellatoidea	7.5	15
E. coli	7.5	15
P. mirabalis	3.25	7.5

Key: MBC/MFC = Minimum Bactericidal Concentration/ Minimum Fungicidal Concentration
MIC = Minimum Inhibitory Concentration



4. CONCLUSION

The result obtained in the present study could justify for the first time the use of the aerial parts of *A. uniflora* in the traditional medicine for the treatment of microbial diseases especially those caused by MRSA, *S. aureus, P. mirabilis, and K. pneumoniae.*

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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